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*Published in:*  
The Journal of Cell Biology

*DOI:*  
[10.1083/jcb.131.6.1453](https://doi.org/10.1083/jcb.131.6.1453)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
1995

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### *Citation for published version (APA):*

Szilard, R. K., Titorenko, V. I., Veenhuis, M., & Rachubinski, R. A. (1995). Pay32p of the Yeast *Yarrowia lipolytica* Is an Intraperoxisomal Component of the Matrix Protein Translocation Machinery. *The Journal of Cell Biology*, 131(6), 1453-1469. <https://doi.org/10.1083/jcb.131.6.1453>

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# Pay32p of the Yeast *Yarrowia lipolytica* Is an Intraperoxisomal Component of the Matrix Protein Translocation Machinery

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**Abstract.** *Pay* mutants of the yeast *Yarrowia lipolytica* fail to assemble functional peroxisomes. One mutant strain, *pay32-1*, has abnormally small peroxisomes that are often found in clusters surrounded by membranous material. The functionally complementing gene *PAY32* encodes a protein, Pay32p, of 598 amino acids (66,733 D) that is a member of the tetratricopeptide repeat family. Pay32p is intraperoxisomal. In wild-type peroxisomes, Pay32p is associated primarily with the inner surface of the peroxisomal membrane, but ~30% of Pay32p is localized to the peroxisomal matrix. The majority of Pay32p in the matrix is complexed with two polypeptides of 62 and 64 kD recognized by antibodies to SKL (peroxisomal targeting signal-1). In contrast, in

peroxisomes of the *pay32-1* mutant, Pay32p is localized exclusively to the matrix and forms no complex. Biochemical characterization of the mutants *pay32-1* and *pay32-KO* (a *PAY32* gene disruption strain) showed that Pay32p is a component of the peroxisomal translocation machinery. Mutations in the *PAY32* gene prevent the translocation of most peroxisome-bound proteins into the peroxisomal matrix. These proteins, including the 62-kD anti-SKL-reactive polypeptide, are trapped in the peroxisomal membrane at an intermediate stage of translocation in *pay32* mutants. Our results suggest that there are at least two distinct translocation machineries involved in the import of proteins into peroxisomes.

**P**EROXISOMES are members of the microbody family of organelles, which also includes the glyoxysomes of plants and the glycosomes of trypanosomes. Peroxisomes are delimited by a single unit membrane and compartmentalize more than 50 enzymes involved in different metabolic functions, most commonly the  $\beta$ -oxidation of fatty acids (Lazarow and de Duve, 1976) and the decomposition of  $H_2O_2$  by catalase (de Duve and Baudhuin, 1966). Peroxisomes are essential for normal human development and physiology, as shown by the lethality of genetic disorders such as Zellweger syndrome in which peroxisome assembly is disrupted (Lazarow and Moser, 1994). Peroxisomes appear to arise by budding and fission of preexisting peroxisomes (Lazarow and Fujiki, 1985), although some evidence for the de novo formation of peroxisomes has been presented (Waterham et al., 1993).

All peroxisomal proteins are synthesized on free polyosomes and imported posttranslationally. Two types of peroxisomal targeting signal (PTS)<sup>1</sup> have been identified in

peroxisomal matrix proteins. Many matrix proteins are targeted to the peroxisome by tripeptide motifs called PTS1, which are identical to or conserved variants of the prototypical Ser-Lys-Leu PTS1 of firefly luciferase (Gould et al., 1987, 1989, 1990; Aitchison et al., 1991; Swinkels et al., 1992). A second type of peroxisomal targeting signal, PTS2, is found at the amino termini of mammalian and yeast thiolases (Swinkels et al., 1991; Osumi et al., 1991; Glover et al., 1994b; Tsukamoto et al., 1994), yeast amine oxidase (Faber et al., 1995), and watermelon malate dehydrogenase (Gietl et al., 1994). A few peroxisomal matrix proteins are targeted by largely uncharacterized internal PTSs (Small et al., 1988; Purdue et al., 1990; Kragler et al., 1993). There is only limited information on the signals that target proteins to the peroxisomal membrane (McCammon et al., 1994). The only protein components of the peroxisomal targeting machinery so far identified are the PTS1 receptors from yeasts and humans (McCollum et al., 1993; Van der Leij et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Nuttley et al., 1995; van der Klei et al., 1995; Terlecky et al., 1995; Wiemer et al., 1995) and the putative PTS2 receptor of the yeast *Saccharomyces cerevisiae* (Marzioch et al., 1994).

Protein import into peroxisomes has been shown (*I*) to

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Rachel K. Szilard and Vladimir I. Titorenko both contributed equally to this work.

1. *Abbreviations used in this paper:* 20K<sub>g</sub>P, 20,000 g pellet enriched for peroxisomes and mitochondria; 20K<sub>g</sub>S, 20,000 g supernatant enriched for

cytosol; PNS, postnuclear supernatant; PTS, peroxisomal targeting signal; TPR, tetratricopeptide repeat.

be energy dependent, requiring ATP hydrolysis but not GTP-hydrolyzing proteins (Imanaka et al., 1987; Rapp et al., 1993; Soto et al., 1993; Wendland and Subramani, 1993); (2) to require cytosolic factors (Wendland and Subramani, 1993), including 70-kD heat shock proteins (Walton et al., 1994); (3) to be dependent on *N*-ethylmaleimide-sensitive proteins of the peroxisomal membrane (Wendland and Subramani, 1993); and (4) to be saturable and competitive (Walton et al., 1992; Wendland and Subramani, 1993; Miura et al., 1992). Peroxisomes also can import oligomeric forms of proteins (Glover et al., 1994a; McNew and Goodman, 1994), which suggests that the peroxisomal translocation machinery can accommodate more than one polypeptide at a time. To date, no component of the peroxisomal translocation machinery itself has been identified. In this paper, we show that the intraperoxisomal protein Pay32p of the yeast *Yarrowia lipolytica* is a component of the peroxisomal translocation machinery for many peroxisomal matrix proteins. We also show that there are at least two distinct pathways for matrix protein translocation across the peroxisomal membrane.

## Materials and Methods

### Strains and Culture Conditions

The yeast strains used in this study are listed in Table I. Strains were grown in complete (YEPD, YPBO) or minimal (YND, YNO) media, as required. Media components were as follows: YEPD: 1% yeast extract, 2% peptone, 2% glucose; YPBO: 0.3% yeast extract, 0.5% peptone, 0.5% K<sub>2</sub>HPO<sub>4</sub>, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 1% Brij 35, 1% (wt/vol) oleic acid; YND: 0.67% yeast nitrogen base without amino acids, 2% glucose; YNO: 0.67% yeast nitrogen base without amino acids, 0.05% (wt/vol) Tween 40, 0.1% (wt/vol) oleic acid. YND and YNO were supplemented with leucine, uracil, lysine and histidine each at 50 µg/ml, as required. Growth was at 30°C unless specified otherwise.

### Isolation of the *pay32* Mutant Strain

The *pay32* strain was isolated after chemical mutagenesis of wild type *Y. lipolytica* E122 cells with 1-methyl-3-nitro-1-nitrosoguanidine (Nuttley et al., 1993). The screening protocol included selection for inability to use oleic acid as a carbon source, immunofluorescence microscopy (Nuttley et al., 1993), fractionation into 20,000 *g* pellet enriched for peroxisomes and mitochondria (20KGP) and 20,000 *g* supernatant enriched for cytosol (20KGS) fractions of yeast cells (Aitchison et al., 1991), and electron microscopy (Waterham et al., 1992; Nuttley et al., 1994). Mutants were characterized by standard genetic techniques for *Y. lipolytica* (Gaillardin et al., 1973).

Table I. *Yarrowia lipolytica* Strains

Strain	Genotype
E122*	<i>MATA, ura3-302, leu2-270, lys8-11</i>
22301-3*	<i>MATB, ura3-302, leu2-270, his1</i>
<i>pay32-1</i> <sup>‡</sup>	<i>MATA, ura3-302, leu2-270, lys8-11, pay32</i>
<i>PAY32</i> <sup>‡</sup>	<i>MATA, ura3-302, leu2-270, lys8-11, pay32, p32GI (LEU2)</i>
<i>pay32-KO</i> <sup>‡</sup>	<i>MATA, ura3-302, leu2-270, lys8-11, pay32::LEU2</i>
<i>pay32-KOB</i> <sup>‡</sup>	<i>MATB, ura3-302, leu2-270, his1, pay32::LEU2</i>
<i>D32WTB</i> <sup>‡</sup>	<i>MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1/+, pay32/+</i>
<i>D32KOB</i> <sup>‡</sup>	<i>MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1/+, pay32/pay32::LEU2</i>

\*C. Gaillardin, Thiverval-Grignon.

<sup>‡</sup>This study.

## Cloning and Characterization of the *PAY32* Gene

The *PAY32* gene was isolated from a *Y. lipolytica* genomic DNA library in the autonomously replicating *Escherichia coli* shuttle vector pINA445 (Nuttley et al., 1993) by functional complementation of the *pay32-1* mutation. Plasmid DNA was introduced into cells by electroporation (Nuttley et al., 1993). *Leu*<sup>+</sup> colonies were screened on YNO-agar plates for restoration of the ability to use oleic acid as the carbon source. Complementary plasmids were recovered by transformation into *E. coli*. Standard recombinant DNA methodologies such as enzymatic modification of DNA, DNA fragment purification, and plasmid purification were performed essentially as described in Ausubel et al. (1989).

### DNA Sequencing

Various restriction endonuclease fragments of the *PAY32* gene were cloned into the vectors pGEM5Zf(+) and pGEM7Zf(+) (Promega, Madison, WI) for dideoxynucleotide sequencing of both strands. The deduced Pay32p amino acid sequence was compared to other known protein sequences using the GENEINFO(R) BLAST Network Service (Blaster) of the National Center for Biotechnology Information (Bethesda, MD).

### Integrative Disruption of the *PAY32* Gene

A plasmid with a genomic DNA insert containing the *PAY32* gene was digested with *Sph*I, and an ~2.3-kbp *Sph*I fragment containing the *Y. lipolytica* *LEU2* gene was ligated into this site. In this way, 1,654 bp of the coding region and 74 bp of the 3' untranslated region of the *PAY32* gene were replaced with the *LEU2* gene. This construct was digested with *Hind*III to liberate the *LEU2* gene flanked by ~1.3 kbp and 1.4 kbp of the 5' and 3' regions of the *PAY32* gene, respectively. This linear molecule was used to transform *Y. lipolytica* E122 and 22301-3 strains to leucine prototrophy. *Leu*<sup>+</sup> transformants that were unable to grow on YNO-agar were further characterized by Southern blotting. *pay32::LEU2* integrants were mated to *pay32-1* and analyzed for complementation.

### Peroxisome Isolation, Subfractionation, and Extraction

Fractionation of oleic acid-induced cells into an organellar pellet (20KGP) enriched for peroxisomes and mitochondria, and a supernatant (20KGS) enriched for cytosol was performed as described previously (Aitchison et al., 1991), except that homogenization of spheroplasts was performed in 5 mM MES (pH 5.5) containing 1 M sorbitol, 1 mM KCl, 0.5 mM EDTA, 0.1% (vol/vol) ethanol, 1 mM PMSF, 1 µg leupeptin/ml, 1 µg pepstatin/ml, 1 µg aprotinin/ml with or without 0.21 mg NaF/ml. Peroxisomes were purified from the 20KGP by isopycnic centrifugation on a discontinuous sucrose gradient (Nuttley et al., 1990). The isolation of peroxisomes by a two-step sucrose flotation gradient was performed after a modification of the procedure of Heyman et al. (1994). Peak fractions (400 µl) of purified peroxisomes isolated by isopycnic centrifugation from wild-type and *pay32* mutant strains were transferred to the bottoms of ultracentrifuge tubes and overlaid first with 2.3 ml of 60% (wt/vol) sucrose then with 2.3 ml of 35% (wt/vol) sucrose. Samples were subjected to centrifugation in a SW50.1 rotor (Beckman Instrs., Inc., Fullerton, CA) at 200,000 *g*<sub>av</sub> for 20 h at 4°C. Gradients were fractionated from the bottom of tubes, and 18 fractions of ~250 µl each were collected. All gradient fractions were assayed for density of sucrose; concentration of protein; and enzymatic activity of catalase, isocitrate lyase, malate synthase, 3-hydroxyacyl-CoA dehydrogenase (peroxisomal marker enzymes), and cytochrome *c* oxidase (mitochondrial marker enzyme). Fractions were also analyzed by immunoblotting with anti-thiolase, anti-SKL, anti-acyl-CoA oxidase, and anti-Pay32p antibodies.

For the subfractionation and extraction of peroxisomes, peroxisomes from the fraction of peak peroxisomal enzymatic activity were lysed by addition of 10 vol of ice-cold Ti8 buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1 mM PMSF, 1 µg leupeptin/ml, 1 µg pepstatin/ml, 1 µg aprotinin/ml; Goodman et al., 1990), followed by incubation on ice for 15 min with occasional agitation. The suspension was centrifuged at 200,000 *g*<sub>av</sub> for 1 h at 4°C in a Beckman TLA 100.2 rotor. The resulting supernatant (*S*<sub>Ti8</sub>) was assayed either for protein and marker enzyme activities or subjected to precipitation of proteins by addition of trichloroacetic acid to 10%, followed by washing of the precipitate in ice-cold 80% (vol/vol) acetone, SDS-PAGE analysis, and immunoblotting. The peroxisome membrane pellet (*P*<sub>Ti8</sub>) was resuspended in ice-cold Ti8 buffer to a final protein concentration of 0.5 mg/ml and subjected to either sodium carbonate extraction (Fujiki et al., 1982) or treatment with 1% sodium deoxycholate; 1%

(vol/vol) Triton X-100; 1 M NaCl; or 1 M urea (final concentrations). After incubation on ice for 45 min with occasional agitation, the samples were centrifuged at 200,000  $g_{av}$  for 1 h at 4°C in a Beckman TLA 100.2 rotor. The resulting pellets were resuspended in ice-cold Ti8 buffer. Proteins from both the supernatant and pellet fractions were analyzed by SDS-PAGE as described above, and gels were either stained with Coomassie blue or their proteins were transferred to nitrocellulose for immunoblotting.

## Antibodies

To produce antibodies to Pay32p, a 1,734-bp fragment of the *PAY32* gene, which contains both open reading frame and 3' untranslated region and codes for amino acids 47-598 of Pay32p, was excised with SphI, made blunt with T4 DNA polymerase, and inserted into the XmnI site of pMAL-c2 (New England Biolabs, Beverly, MA) in-frame and downstream of the open reading frame encoding the maltose-binding protein. Antibodies to the fusion protein were raised in guinea pig and rabbit as described previously (Eitzen et al., 1995). Anti-Pay32p antibodies were affinity purified as described by Crane et al. (1994).

Rabbit antibodies to *S. cerevisiae* peroxisomal acyl-CoA oxidase were a generous gift of Dr. Joel Goodman (University of Texas, Southwestern Medical Center, Dallas, TX). Anti-SKL antibodies were raised in rabbit against the peptide NH<sub>2</sub>-CRYHLKPLQSKL-CO<sub>2</sub>H linked to keyhole limpet hemocyanin as described previously (Aitchison et al., 1992). Antibodies to *Y. lipolytica* peroxisomal thiolase were generated by injection of a thiolase-maltose-binding protein fusion into guinea pig and rabbit.

## Immunoaffinity Chromatography

Covalent coupling of affinity-purified rabbit anti-SKL and anti-Pay32p antibodies to protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) and immunoaffinity chromatographic selection of peroxisomal matrix proteins were performed as described by Voos et al. (1994) with the following modifications. The  $S_{T18}$  containing peroxisomal matrix proteins was diluted with an equivolume of 90 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM PMSF, 1  $\mu$ g leupeptin/ml, 1  $\mu$ g pepstatin/ml, 1  $\mu$ g aprotinin/ml. The diluted  $S_{T18}$  (500  $\mu$ l) was cleared of any nonspecifically binding proteins by incubation for 20 min at 4°C with protein A-Sepharose (50  $\mu$ l wet volume washed five times in 10 mM Tris-HCl [pH 7.5]). The cleared solution was then subjected to immunoaffinity chromatography. Bound proteins were washed four times in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (vol/vol) Triton X-100, and eluted with 100 mM glycine (pH 2.8). Proteins were precipitated by addition of trichloroacetic acid to 10%, washed in ice-cold 80% (vol/vol) acetone, and then subjected to SDS-PAGE followed by immunoblotting.

For immunoprecipitation of SDS-denatured peroxisomal matrix proteins, the  $S_{T18}$  was adjusted to 1% SDS, heated at 65°C for 20 min, and processed for immunoprecipitation with anti-SKL and anti-Pay32p antibodies as described (Franzoso et al., 1991).

## Protease Protection

A 20K $gP$  was prepared as described above, except that protease inhibitors were omitted. The 20K $gP$  was gently resuspended in homogenization buffer (1 M sorbitol, 5 mM MES [pH 5.5]). Aliquots containing 240  $\mu$ g of protein were incubated with 0, 10, 20, or 50  $\mu$ g of trypsin for 40 min on ice, either in the presence or absence of Triton X-100 at 0.5% (vol/vol) final concentration. The reaction was terminated by addition of trichloroacetic acid to 10% final concentration. The protein precipitates were washed with ice-cold 80% (vol/vol) acetone, and equivalent fractions of each reaction were subjected to SDS-PAGE, followed by immunoblotting with either anti-thiolase, anti-SKL, or anti-Pay32p antibodies.

## Immunofluorescence

Immunofluorescence microscopy was performed as described by Aitchison et al. (1992), except that spheroplasts were fixed according to Pringle et al. (1991). Rabbit anti-Pay32p antibodies were affinity purified for immunofluorescence as follows. Whole cell lysates of the *pay32-KO* mutant grown in YPBO were made in SDS-PAGE sample buffer (2% SDS, 10% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue) as described (Franzoso et al., 1991). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Strips of membrane (1 cm  $\times$  3 cm) were incubated in 100 mM Tris-HCl, pH 7.0, 6 M guanidine-HCl, 50 mM NaCl for 5 min with gentle agitation at room temperature. After three washes with water (5 min

each), the strips were incubated in PBS with 3.7% (vol/vol) formaldehyde for 5 min with gentle agitation at room temperature. The strips were then washed three times with water (5 min each), blocked with PBS containing 1% nonfat dry milk and incubated with anti-Pay32p antiserum at dilutions of 1:50, 1:100, and 1:200 in PBS containing 1% nonfat dry milk (~250  $\mu$ l of diluted antiserum was used for each strip) for 30 min with gentle agitation at room temperature. The purified anti-Pay32p antibodies were recovered and used for immunofluorescence microscopy.

## Analytical Procedures

Whole cell lysates were prepared as described by Nuttley et al. (1993). Enzymatic activities of catalase (Luck, 1963), isocitrate lyase (Barth and Weber, 1987), malate synthase (Dixon and Kornberg, 1962), 3-hydroxyacyl-CoA dehydrogenase (Osumi and Hashimoto, 1979), and cytochrome *c* oxidase (Douma et al., 1985) were determined by established methods. SDS-PAGE (Laemmli, 1970) and immunoblotting using a semidry electrophoretic transfer system (model ET-20, Tyler Research Instruments, Edmonton, AB) (Kyhse-Andersen, 1984) were performed as described. Antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Life Sciences, Oakville, ON). Densitometry was performed with a LKB Ultrosan XL laser densitometer (LKB Instruments, Bromma, Sweden). Quantification of densitometric signals was done under conditions in which the signal was proportional to the antigen concentration. Protein concentration was determined with a protein assay kit (BioRad Laboratories, Mississauga, ON) using bovine serum albumin as standard. Protein quantification of peroxisomal subfractions was also performed by the method of Schaffner and Weissmann (1973).

## Results

### The *pay32-1* Mutant Has Abnormal Peroxisome Morphology

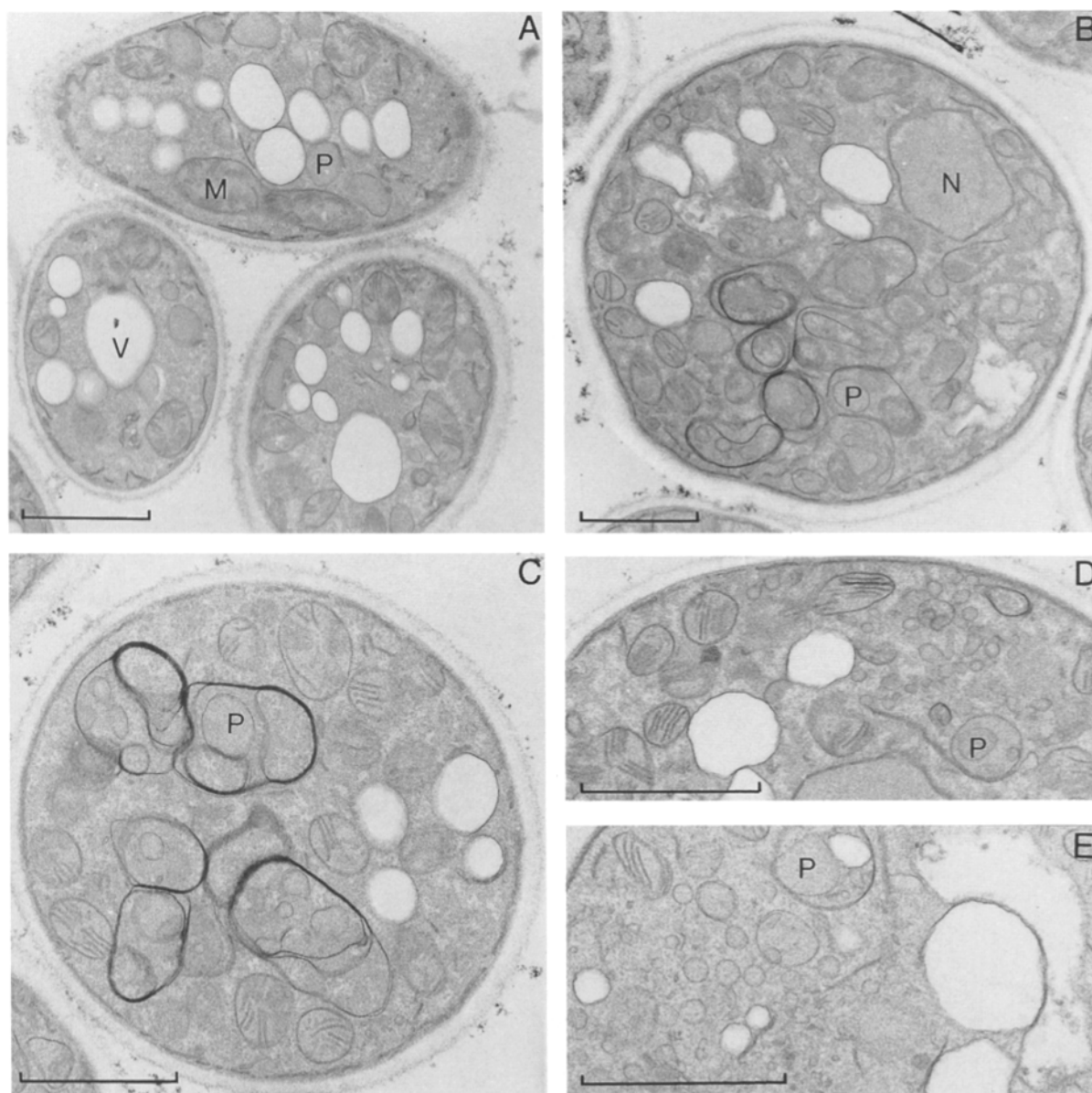
The mutant *pay32-1* was initially identified by its inability to grow on media containing oleic acid as the sole carbon source (data not shown). Biochemical characterization showed that *pay32-1* had deficiencies in the targeting and import of several peroxisomal matrix proteins (detailed discussion follows), which conforms to the classical *pay* mutant phenotype.

Electron microscopic analysis of wild-type cells grown in oleic acid-containing (YPBO) medium showed large round peroxisomes well separated from one another and delimited by single membranes (Fig. 1 A). In contrast, peroxisomes from *pay32-1* cells were generally smaller than those of wild-type cells and were often found in clusters surrounded by electron-dense membranous material of unknown composition (Fig. 1 B), although loose peroxisomes were also present (Fig. 1 D). The large membranous structures surrounding peroxisomes in *pay32-1* cells appear not to be derived from vacuolar, endoplasmic reticular, or Golgi membranes (data not shown).

Immunoelectronmicroscopical analysis showed strong labeling of wild-type peroxisomes with anti-thiolase (Fig. 2 E) and anti-SKL (Fig. 2 F) antibodies. Thiolase and anti-SKL-reactive proteins were also detected in the membranous complexes surrounding peroxisomes of the *pay32-1* mutant (arrows in Fig. 2, A and B, respectively). A large immunolabeled peroxisome outside the membranous complex can be seen in Fig. 2 B (P).

### Cloning and Analysis of the *PAY32* Gene

The *PAY32* gene was isolated from a *Y. lipolytica* genomic DNA library in the autonomously replicating *E. coli* shuttle vector pINA445 (Nuttley et al., 1993) by functional



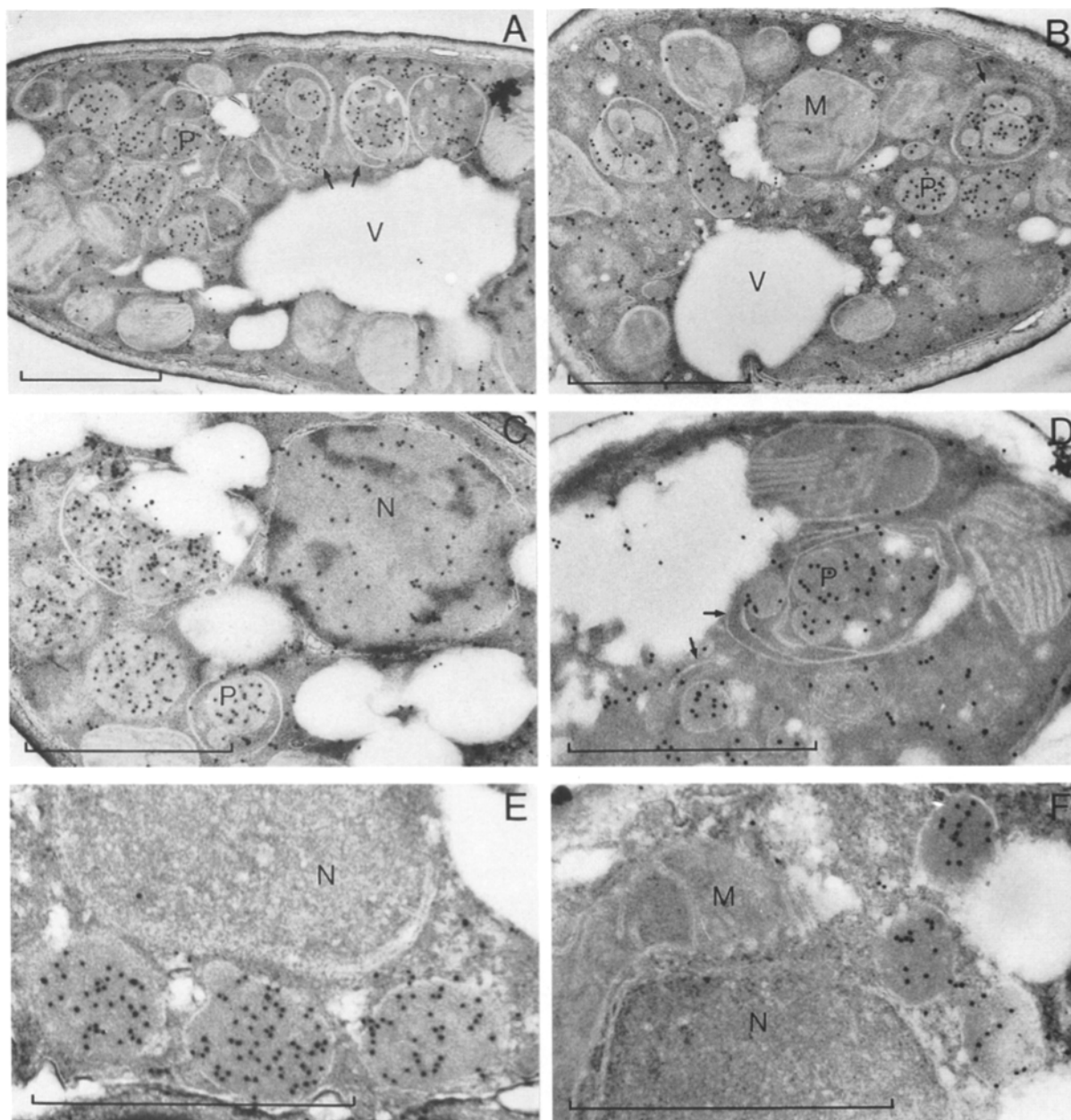
**Figure 1.** Ultrastructure of the wild-type and *pay32* mutant strains. The wild-type (A), *pay32-1* (B and D), and *pay32-KO* (C and E) strains were grown to saturation in YEPD medium, transferred to YPBO medium, and grown for an additional 8 h in YPBO medium. Cells were fixed in  $\text{KMnO}_4$  and processed for electron microscopy. P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole. Bar, 1  $\mu\text{m}$ .

complementation of the mutation in *pay32-1* cells. Of the  $5 \times 10^3$  transformants screened, one strain, *PAY32*, was found to have restored growth on oleic acid (data not shown). The strain *PAY32* carried the plasmid p32G1, which contains an  $\sim 5.4$ -kbp insert of *Y. lipolytica* genomic DNA. An  $\sim 4.1$ -kbp region and an overlapping  $\sim 3.5$ -kbp region within this insert were sufficient to functionally complement the *pay32-1* mutation (Fig. 3 a). Sequencing within the 3.5-kbp region revealed a 1,794-bp open reading frame encoding a 598-amino acid protein, Pay32p, with a predicted molecular weight of 66,733 (Fig. 3 b).

The sequence of Pay32p shows extensive homology to a family of proteins shown to be essential for peroxisome biogenesis in yeasts and humans: *Pichia pastoris* Pas8p (McCollum et al., 1993), *S. cerevisiae* Pas10p (Van der Leij

et al., 1993), *Hansenula polymorpha* Pah2p/Per3p (Nuttley et al., 1995; van der Klei et al., 1995), and human Pxr1p (Dodt et al., 1995; Fransen et al., 1995; Wiemer et al., 1995). These proteins all share a common structural feature, the tetratricopeptide repeat (TPR) motif, which is a degenerate repeat of 34 amino acids believed to form an interlocking series of  $\alpha$ -helices (Goebel and Yanagida, 1991). The region of greatest homology among these proteins lies in their TPR domains (data not shown).

It should be noted that the synthesis of Pas8p in *pay32-1* cells did not restore growth on oleic acid. Similarly, synthesis of Pay32p in *pas8-1* cells did not restore growth on oleic acid. In both cases, the heterologous protein was detectable in immunoblots of whole cell extracts (data not shown). One possible explanation for a negative result of



**Figure 2.** Immunocytochemical analysis of wild-type and *pay32* mutant strains. The wild-type (*E* and *F*), *pay32-1* (*A* and *B*) and *pay32-KO* (*C* and *D*) strains were grown as described in Fig. 1. The cells were fixed with glutaraldehyde/formaldehyde and processed for immunoelectronmicroscopy with anti-thiolase (*A*, *C*, and *E*) or anti-SKL (*B*, *D*, and *F*) antibodies. Symbols as in Fig. 1. Arrows point to membranous complexes.

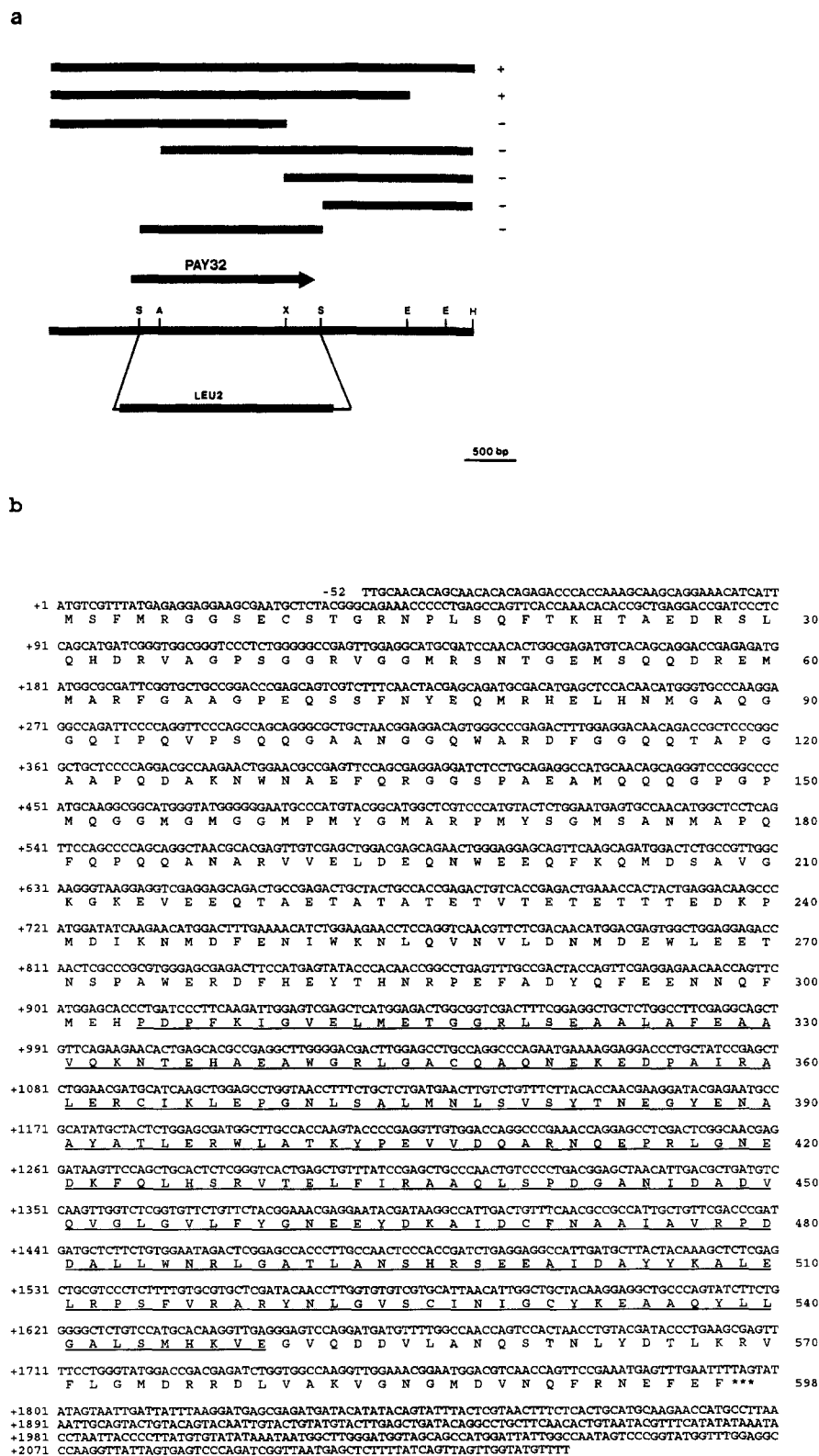
this type is that Pas8p and Pay32p do not perform the same function.

Integrative disruption of the *PAY32* gene with the *Y. lipolytica* *LEU2* gene was used to create the strains *pay32-KO* and *pay32-KOB* in the A and B mating types, respectively. Strains containing a disrupted *PAY32* gene were unable to grow on oleic acid (data not shown) and had the same peroxisomal morphology (Figs. 1, *C* and *E*, 2, *C* and *D*) and peroxisomal protein targeting and import defects (see below) as the original *pay32-1* mutant. The diploid strain *D32WTB*, resulting from the mating of the *pay32-1* strain and the wild-type strain 22301-3, could grow on oleic

acid, demonstrating the recessive nature of the *pay32-1* mutation. A diploid strain resulting from the mating of *pay32-1* and *pay32-KOB*, *D32KOB*, was unable to grow on oleic acid (data not shown), indicating that the authentic *PAY32* gene had been cloned.

Expression of the *PAY32* gene is inducible by growth on oleic acid. A small amount of Pay32p is present in wild-type cells grown in glucose-containing medium. Shifting cells to oleic acid-containing medium causes an increase in the level of Pay32p that reaches steady state 3 h after the shift at a level 10 times that found in glucose-grown cells (data not shown).





**Figure 3.** Cloning and analysis of the *PAY32* gene. (a) Complementing activity of inserts containing the *PAY32* gene. The longest line indicates the 4.1-kbp complementing fragment from the original insert in p32G1. The arrow shows the direction and position of the open reading frame of the *PAY32* gene. The (+) symbol denotes the ability of an insert to confer growth on oleic acid to *pay32-1*. The (-) sign denotes the inability of an insert to confer growth on oleic acid to *pay32-1*. The site of integration of *Y. lipolytica LEU2* in the *PAY32* gene is shown at the bottom. A, ApaI; E, EcoRI; H, HindIII; S, SphI; X, XhoI. (b) Nucleotide sequence of the *PAY32* gene and deduced amino acid sequence of Pay32p. The region of TPR motifs is underlined. The nucleotide sequence reported in this paper has been submitted to GenBank with accession number U28155.

### Mutations in the *PAY32* Gene Affect the Subcellular Localization but Not the Synthesis of Peroxisomal Proteins

To determine the effects of the *pay32-1* and *pay32-KO* mutations on the level of synthesis of peroxisomal pro-

teins, the wild-type and mutant strains were grown first in glucose-containing (YEPD) medium for 10 h, and then shifted to oleic acid-containing medium (YPBO) for an additional 9 h. Under these conditions, the levels of peroxisomal matrix proteins analyzed (i.e., catalase, isocitrate lyase, malate synthase, 3-hydroxyacyl-CoA dehydroge-

nase, acyl-CoA oxidase, thiolase, and two polypeptides reactive to anti-SKL [PTS1] antibodies) were greatly increased and reached steady state in the wild-type strain (data not shown). The steady-state levels of these proteins in both the *pay32-1* and *pay32-KO* mutants were unchanged vis-à-vis the levels in the wild-type strain (Fig. 4, *top panel* [catalase (CAT), isocitrate lyase (ICL), malate synthase (MLS), 3-hydroxyacyl-CoA dehydrogenase (HAD)]; Fig. 6, *a* and *b*, thiolase; Fig. 8 *a*, acyl-CoA oxidase; Fig. 8 *b*, anti-SKL reactive polypeptides). However, the distribution of these proteins between a 20,000 g pellet (20KgP) enriched for peroxisomes and mitochondria and a 20,000 g supernatant (20KgS) enriched for cytosol was altered in both mutants as compared to the wild-type strain. A significant fraction of peroxisomal matrix proteins in the wild-type strain was associated with the 20KgP, while in the mutant strains these proteins were predominantly localized to the 20KgS (Fig. 4, *middle and bottom panels*, respectively; Fig. 6, *a* and *b*; Fig. 8, *a* and *b*).

Peroxisomes were isolated from the wild-type and mutant strains to determine which peroxisome functions were affected by mutations in the *PAY32* gene. In the wild-type

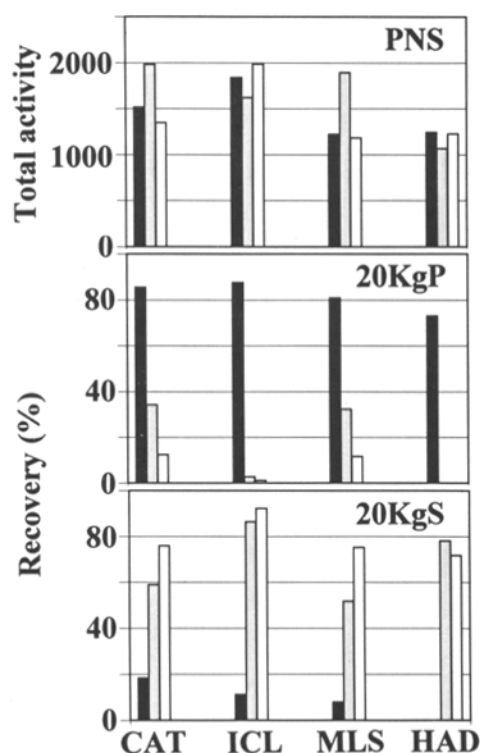
strain, catalase, isocitrate lyase, and malate synthase activities were found primarily in fractions 1 to 6, with peaks of activity in fraction 4 (Fig. 5 *a*). Immunoblotting showed that thiolase and anti-SKL-reactive polypeptides were also localized to fractions 1 to 6, with the most intense signals in fraction 4 (Fig. 5, *b* and *c*, respectively). Fraction 4 had a density of 1.21 g/cm<sup>3</sup>. Since all six peroxisomal proteins colocalized to fractions 1 to 6 and as there was essentially no cytochrome *c* oxidase activity in these fractions, peroxisomes free of mitochondrial contamination were purified in fractions 1 to 6 from the parental strain. *Pay32p* is also a peroxisomal protein, as it is enriched in the same fractions as are the other peroxisomal proteins (Fig. 5 *d*). A similar analysis of the *pay32-1* and *pay32-KO* mutants showed a peak of peroxisomal proteins at a density 1.24 g/cm<sup>3</sup> (fraction 2, see Fig. 5). Peroxisome-associated proteins constituted 26.2%, 12.4%, and 3.8% of the 20KgPs of the wild-type, *pay32-1* and *pay32-KO* strains, respectively. The reduced recovery of peroxisome-associated proteins in the *pay32* mutant strains may be due to the fact that only single peroxisomes, and not the electron-dense membranous structures surrounding them, are localized to the 20KgP upon subcellular fractionation. However, this is unknown at present.

The fractions most highly enriched for peroxisomes were further analyzed by sucrose-flotation (Heyman et al., 1994). All peroxisomal marker proteins tested floated out of the most dense sucrose and concentrated at the interface between 60 and 35% sucrose at densities of 1.22 g/cm<sup>3</sup> and 1.25 g/cm<sup>3</sup> for the parental and mutant strains, respectively (data not shown). Therefore, the colocalization of peroxisomal marker proteins in the wild-type strain and in the *pay32-1* and *pay32-KO* mutant strains was not due to the formation of protein aggregates.

#### *A Subset of Peroxisome-associated Proteins Is Translocated Normally into the Matrix of Peroxisomes of pay32 Mutants*

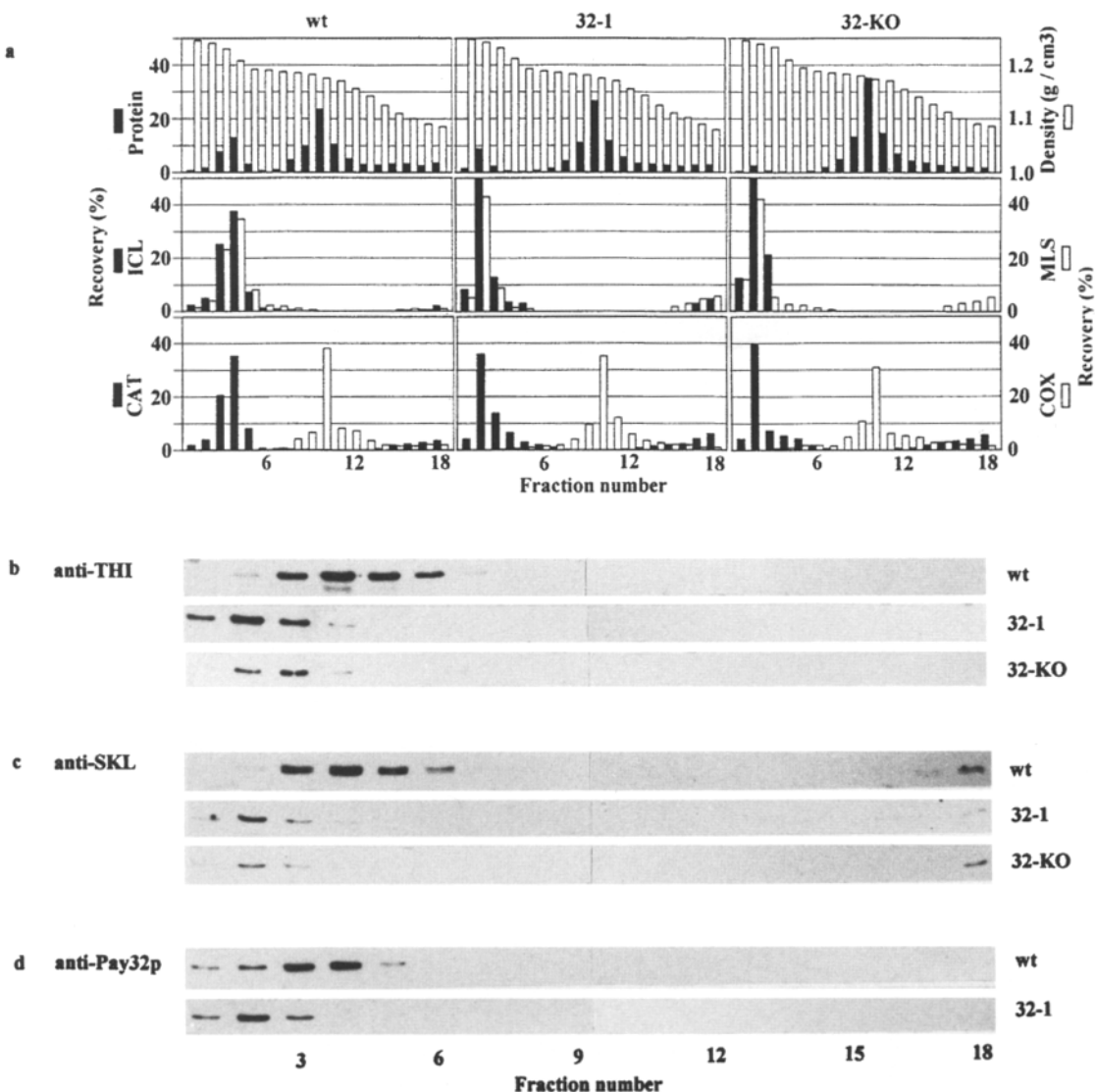
Immunoblot analysis of equal quantities of protein from highly purified peroxisomes revealed that thiolase was present in the peroxisomes of the *pay32-1* and *pay32-KO* strains at levels comparable to that found in peroxisomes of the wild-type strain (Fig. 6 *c*, lanes *PER*). In peroxisomes from all three strains, thiolase was localized exclusively to the peroxisomal matrix. Subfractionation of peroxisomes showed that thiolase was found only in a 200,000 g supernatant isolated from peroxisomes lysed with Ti8 buffer, which is enriched for peroxisomal matrix proteins (Fig. 6 *c*, lanes *S<sub>Ti8</sub>*), and protease-protection experiments showed that thiolase in the *pay32-KO* strain was resistant to protease digestion in the absence of detergent, but was completely digested when the peroxisomal membrane was disrupted with Triton X-100 (Fig. 6 *d*). Unlike thiolase found in the peroxisomes of the wild-type strain, thiolase in the peroxisomal matrix of the *pay32-1* and *pay32-KO* strains was not proteolytically processed to its mature form (Fig. 6 *c*). Therefore, thiolase translocation across the peroxisomal membrane does not require proteolytic processing of the thiolase precursor to mature thiolase.

We have measured the specific activities of catalase (Fig. 6, *e* and *f*) and malate synthase (Fig. 6, *g* and *h*) in



**Figure 4.** Peroxisomal proteins are induced normally by oleic acid but are mislocalized to the cytoplasm in *pay32* mutant strains. Wild-type (solid bar), *pay32-1* (stippled bar), and *pay32-KO* (open bar) strains were grown for 10 h in YEPD medium, transferred to YPBO medium, and grown for an additional 9 h. Cells were subjected to subcellular fractionation as described in Materials and Methods. The total enzymatic activities of catalase (CAT), isocitrate lyase (ICL), malate synthase (MLS), and 3-hydroxyacyl-CoA dehydrogenase (HAD) in 50 mg of protein of postnuclear supernatant (PNS) are expressed either in U (CAT), mU (ICL, MLS), or mU  $\times$  10 (HAD) (*top panel*). The percentages of total enzymatic activity recovered in the 20KgP (*middle panel*) and 20KgS (*bottom panel*) are shown.





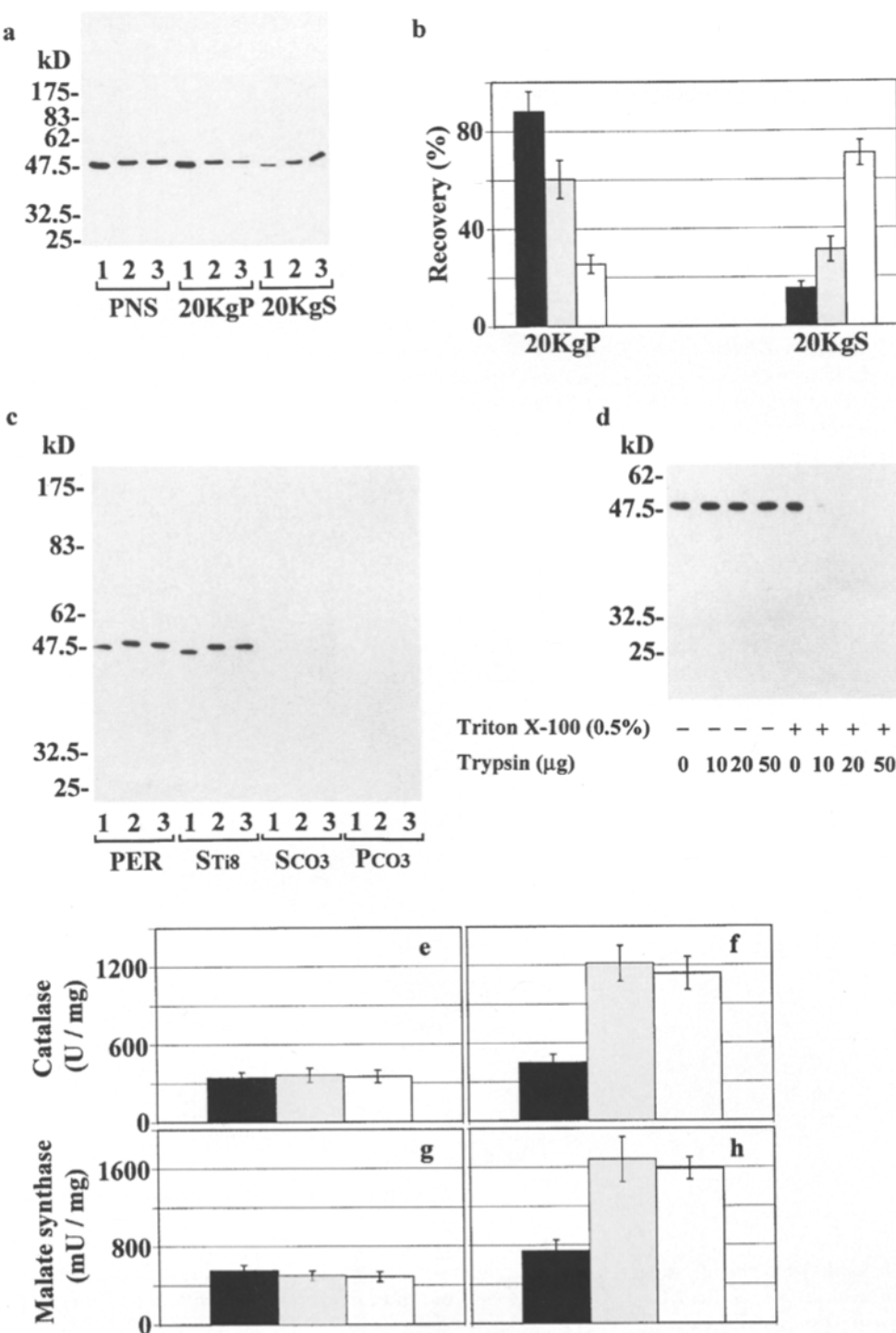
**Figure 5.** Purification of peroxisomes and immunolocalization of proteins. Wild-type (wt), *pay32-1* (32-1), and *pay32-KO* (32-KO) strains were grown as described in Fig. 4. Fractions enriched for peroxisomes were isolated as described in Materials and Methods. For each strain, 9  $\mu$ g of protein was loaded onto the gradient. (a) Sucrose density (g/cm<sup>3</sup>) and percent recovery of loaded protein and of enzymatic activities in gradient fractions. COX, cytochrome c oxidase. Other abbreviations as in Fig. 4. Gradient fractions were analyzed by immunoblotting with anti-thiolase (b, anti-THI), anti-SKL (c), and anti-Pay32p (d) antibodies. In b and c, 2% and in d, 10% of the volume of each fraction was analyzed.

highly purified peroxisomes from wild-type, *pay32-1* and *pay32-KO* cells. No significant differences in specific activities calculated per mg of total peroxisomal protein were found among the three strains (Fig. 6, e and g), suggesting that both catalase and malate synthase, when targeted (bound) to the peroxisome, were translocated normally into the matrix in the *pay32-1* and *pay32-KO* strains. Peroxisome subfractionation experiments also showed that 78–89% of total catalase and malate synthase activities were found in the S<sub>T18</sub> enriched for peroxisomal matrix proteins (data not shown). It is noteworthy that the specific activities of both catalase and malate synthase, when calculated per mg of peroxisomal matrix protein, were 2–3 times higher in the *pay32-1* and the *pay32-KO* strains as compared to the values for the wild-type strain (Fig. 6, f and h). Therefore, some peroxisome-associated matrix

proteins are not efficiently translocated into peroxisomes of the *pay32* mutants, and the relative levels of correctly translocated proteins, such as catalase and malate synthase, are selectively enriched in the peroxisomal matrix of the mutant strains.

#### **Most Matrix Proteins Bound to the Peroxisome Cannot be Translocated Across the Peroxisomal Membrane in the *pay32* Mutants**

Subfractionation of peroxisomes from the wild-type strain showed that  $76.9 \pm 5.0\%$  of total peroxisomal protein was found in the S<sub>T18</sub> enriched for matrix proteins (Fig. 7 c, solid bar). In contrast, peroxisomal matrix proteins composed  $28.8 \pm 1.7$  and  $29.9 \pm 2.3\%$  of total peroxisomal protein in the *pay32-1* (stippled bar) and *pay32-KO* (open



was incubated with 0, 10, 20, and 50 μg of trypsin in the absence (–) or presence (+) of 0.5% (vol/vol) Triton X-100 for 40 min on ice. Reactions were terminated by addition of trichloroacetic acid to 10%. Equal fractions of the samples were subjected to immunoblot analysis with anti-thiolase antibodies. (e–h) Specific activities of catalase (e and f) and malate synthase (g and h) in the wild-type (solid bar), *pay32-1* (stippled bar), and *pay32-KO* (open bar) strains. Activities were measured in whole peroxisomes (e and g) and in the  $S_{Ti8}$  enriched for peroxisomal matrix proteins (f and h). Activities were calculated either per mg of total peroxisomal protein (e and g) or per mg of peroxisomal matrix protein (f and h). The values reported are the means ± the standard deviation from three independent experiments.

*bar*) strains, respectively. Treatment of  $P_{Ti8}$  (see legend to Fig. 6 c) with 0.1 M  $Na_2CO_3$  (pH 11), followed by recentrifugation at 200,000  $g_{av}$  for 1 h, showed that in all three strains, most of the protein was recovered in a pellet ( $P_{CO3}$ )

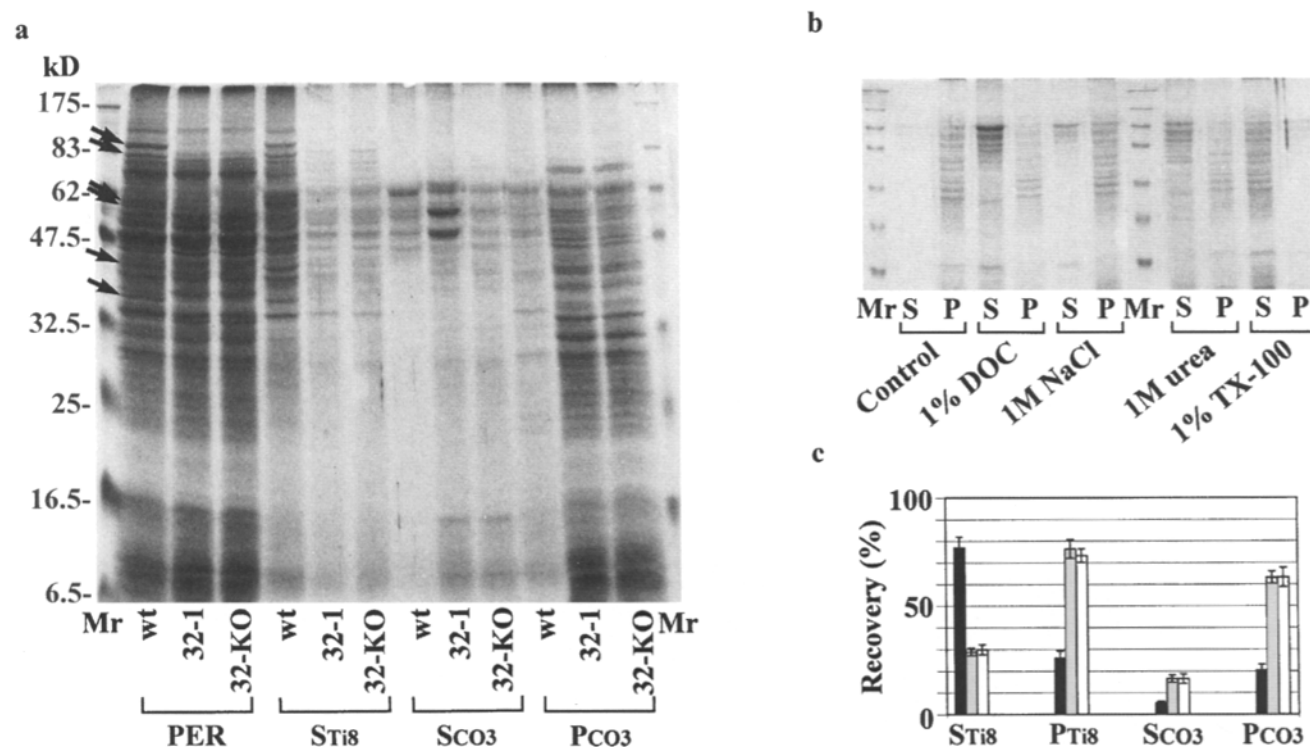
enriched for proteins that are tightly associated with the peroxisomal membrane. However, the amounts of these tightly associated membrane proteins relative to the total peroxisomal protein isolated from cells of the parental

**Figure 6.** Mutations in the *PAY32* gene do not impair the import of thiolase, catalase, and malate synthase into the peroxisomal matrix but do prevent proteolytic processing of the thiolase precursor. (a) Equal fractions (0.2% of the total volume) of the postnuclear supernatant (PNS), 20KgP and 20KgS from the wild-type, *pay32-1* and *pay32-KO* strains were analyzed by immunoblotting with anti-thiolase antibodies. (b) Quantitative analysis of the recovery of thiolase in the 20KgP and 20KgS for the wild-type (solid bar), *pay32-1* (stippled bar), and *pay32-KO* (open bar) strains. Immunoblots as in a were scanned densitometrically. Values reported were standardized to the signals obtained for the PNS of the corresponding strain and are the means ± the standard deviation of three independent experiments. (c) Immunoblot analysis with anti-thiolase antibodies. Peroxisomes from the wild-type (lanes 1), *pay32-1* (lanes 2), and *pay32-KO* (lanes 3) strains were subjected to subfractionation as described in Materials and Methods. Whole peroxisomes (20 μg of protein) were used as starting material for subfractionation. PER, whole peroxisomes (20 μg);  $S_{Ti8}$ , 200,000  $g$  supernatant recovered after peroxisome lysis with Ti8 buffer (step 1);  $S_{CO3}$  and  $P_{CO3}$ , 200,000  $g$  supernatant and pellet, respectively, recovered after treatment of the 200,000  $g$  pellet from step 1 ( $P_{Ti8}$ ) with 0.1 M  $Na_2CO_3$  (pH 11). (d) Protease protection analysis. The  $P_{Ti8}$  fraction (240 μg of protein) of the *pay32-KO* mutant strain

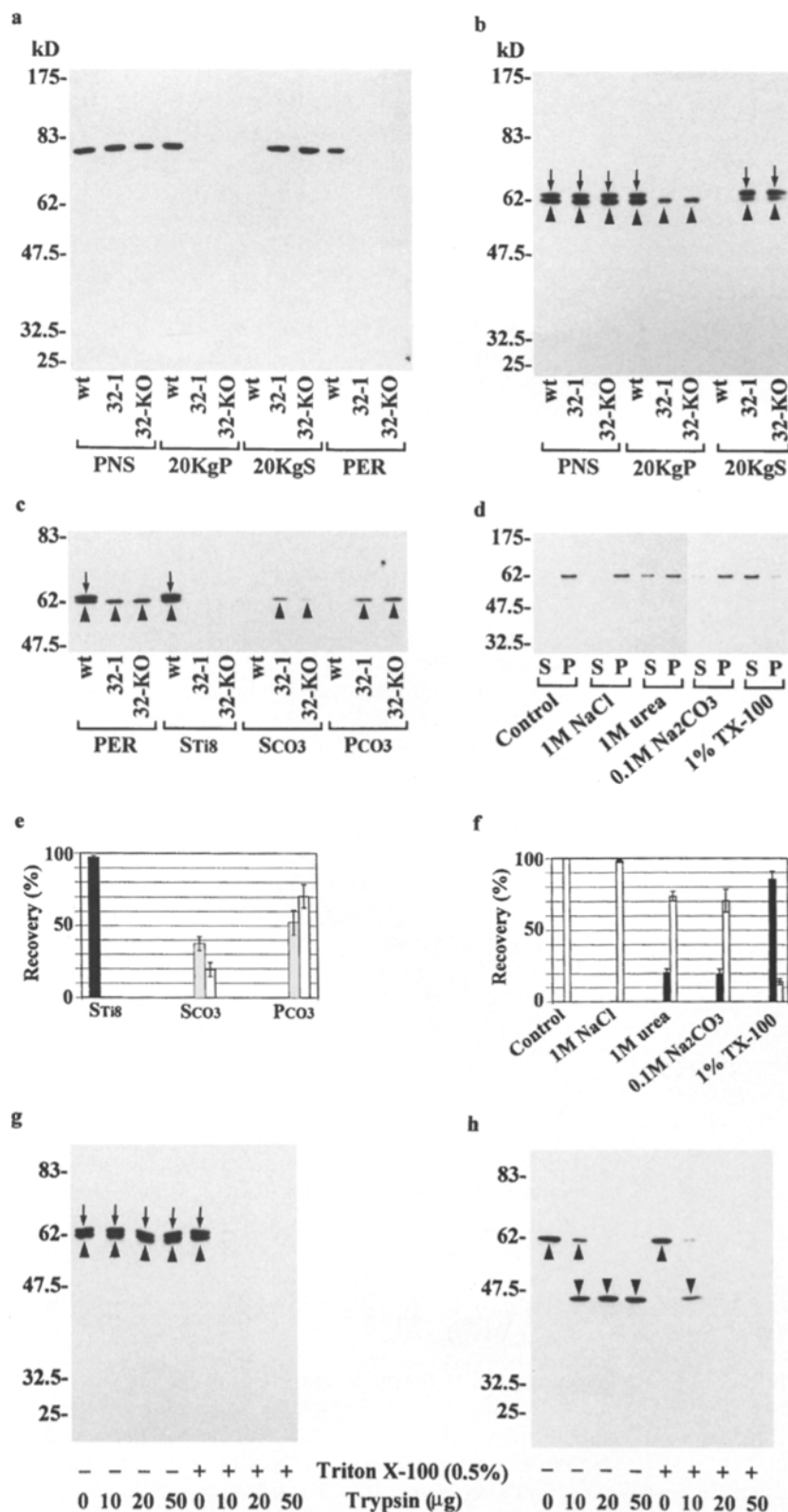
strain ( $20.3 \pm 2.7\%$  of total peroxisomal protein) were significantly lower than in cells from the mutant strains *pay32-1* and *pay32-KO* ( $63.1 \pm 2.9\%$  and  $63.2 \pm 4.5\%$ , respectively; Fig. 7 c). This shifting of matrix proteins to the peroxisomal membrane in the *pay32* mutant strains was confirmed by SDS-PAGE analysis of the various peroxisome subfractions (Fig. 7 a). Only a few proteins in whole peroxisomes of the wild-type strain (Fig. 7 a, lanes *PER*, arrows) were missing from the whole peroxisomes of the *pay32-1* and *pay32-KO* mutants, when equal amounts of total peroxisomal proteins were analyzed. Most peroxisomal proteins were found in comparable amounts in peroxisomes of all three strains (Fig. 7 a, lanes *PER*). In the wild-type strain, most peroxisomal proteins were recovered in the matrix fraction (Fig. 7 a, *S*<sub>Ti8</sub>, lane *wt*), while in the *pay32-1* and *pay32-KO* strains, most peroxisomal proteins were found tightly associated with the peroxisomal membrane (Fig. 7 a, *P*<sub>CO3</sub>, lanes *32-1* and *32-KO*, respectively). This tight association of the majority of peroxisomal proteins with the peroxisomal membrane in the mutant strains could also be shown when peroxisomal membrane fractions (*P*<sub>Ti8</sub>) were extracted under conditions that solubilize preferentially peripheral membrane proteins (1 M NaCl and 1 M urea) or all membrane proteins (1% Triton X-100). Fig. 7 b shows that membrane-associated proteins

from peroxisomes of *pay32-KO* cells were tightly associated with membrane, as they could be released completely into the supernatant fraction (S) only by treatment with 1% Triton X-100. It is unclear why three proteins of molecular masses 30–35 kD could not be solubilized by 1% sodium deoxycholate. It is possible that these three proteins, once solubilized by sodium deoxycholate, quickly form insoluble aggregates.

A 62-kD anti-SKL-reactive polypeptide was found among the proteins tightly associated with the peroxisomal membrane in both the *pay32-1* and *pay32-KO* mutants (Fig. 8 c, *P*<sub>CO3</sub>, lanes *32-1* and *32-KO*, upward pointing arrowheads; Fig. 8 e, *stippled and open bars*, respectively). In peroxisomes isolated from the parental strain, the 62-kD polypeptide was exclusively associated with the peroxisomal matrix (Fig. 8 c, *S*<sub>Ti8</sub>, lane *wt*, upward pointing arrowhead; Fig. 8 e, *solid bar*). Treatment of the peroxisomal membrane fraction *P*<sub>Ti8</sub> from the *pay32-KO* strain under conditions that solubilize peripheral membrane proteins (1 M NaCl, 1 M urea, 0.1 M Na<sub>2</sub>CO<sub>3</sub>) released some of the 62-kD polypeptide to the soluble (Fig. 8 d, lanes S; Fig. 8 f, *solid bars*) fraction, while most of the polypeptide could be solubilized only by treatment with 1% Triton X-100. Protease-protection experiments confirmed that the 62-kD polypeptide was exclusively localized to the matrix of peroxisomes iso-



**Figure 7.** Mutations in the *PAY32* gene prevent translocation of the bulk of peroxisomal matrix proteins across the peroxisomal membrane. (a) Coomassie blue-stained SDS-polyacrylamide gel of whole peroxisomes (*PER*, 60  $\mu$ g of protein) and peroxisome subfractions (*S*<sub>Ti8</sub>, *S*<sub>CO3</sub>, and *P*<sub>CO3</sub>) of the wild-type (*wt*), *pay32-1* (*32-1*), and *pay32-KO* (*32-KO*) strains. Abbreviations are as in Fig. 6 a. Subfractions were isolated from 60  $\mu$ g of starting purified peroxisomes. (b) The *P*<sub>Ti8</sub> of the *pay32-KO* strain was resuspended in Ti8 buffer, and equal aliquots were treated with one of 1% sodium deoxycholate, 1 M NaCl, 1 M urea, or 1% (wt/vol) Triton X-100. After incubation on ice for 45 min, all samples were separated into supernatant (S) and pellet (P) fractions by centrifugation at 200,000 g for 1 h at 4°C, subjected to SDS-PAGE, and stained with Coomassie blue. Control, sample incubated on ice with Ti8 buffer alone. (c) Peroxisomes isolated from the wild-type (*solid bar*), *pay32-1* (*stippled bar*), and *pay32-KO* (*open bar*) strains were subjected to subfractionation, and the percentage of total peroxisomal protein recovered in each subfraction was determined. The values reported are the means  $\pm$  the standard deviation from three independent experiments.



**Figure 8.** Mutations in the *PAY32* gene prevent the targeting of acyl-CoA oxidase and a 64-kD anti-SKL reactive polypeptide to peroxisomes and impair the translocation, but not targeting, of a 62-kD anti-SKL reactive polypeptide into peroxisomes. Equal portions (0.2% of the total volume) of the postnuclear supernatant (PNS), the 20KgP and the 20KgS and 10 μg of purified peroxisomes from each of the wild-type and *pay32-1* and *pay32-KO* strains were analyzed by immunoblotting with anti-acyl-CoA oxidase (a) and anti-SKL (b) antibodies. (c) Immunodetection of anti-SKL reactive polypeptides in peroxisomal subfractions. PER lanes contained 20 μg of purified peroxisomes. Peroxisomal subfractions were isolated from 20 μg of starting purified peroxisomes. (e) Recovery of anti-SKL polypeptides in the various peroxisomal subfractions from the wild-type (solid bar), *pay32-1* (stippled bar), and *pay32-KO* (open bar) strains. The percentage of total peroxisomal protein recovered in each subfraction is reported. Values are the means ± the standard deviation of three independent experiments. (d) The  $P_{T18}$  from the *pay32-KO* strain was treated with various agents and divided into supernatant (S) and pellet (P) fractions as in Fig. 7 b. The control was treated with Ti8 buffer alone. S and P fractions were subjected to SDS-PAGE and immunoblotted with anti-SKL antibodies. (f) Recovery of the 62-kD anti-SKL reactive polypeptide in the S (solid bar) and P (open bar) fractions of d. Localization of anti-SKL reactive polypeptides in peroxisomes of the wild-type (g) and *pay32-KO* (h) strains by protease protection analysis. Protease protection was carried out as in Fig. 6 b. Arrow, 64-kD anti-SKL reactive polypeptide; upward pointing arrowhead, 62-kD anti-SKL reactive polypeptide; downward pointing arrowhead, 45-kD trypsin-resistant fragment.

lated from the wild-type strain (Fig. 8 g, upward pointing arrowheads). In contrast, the 62-kD polypeptide was sensitive to protease even in the absence of detergent when peroxisomes were isolated from *pay32-KO* cells (Fig. 8 h, upward pointing arrowheads), and protease cleavage re-

sulted in a smaller protease-resistant fragment of ~45 kD (Fig. 8 h, downward pointing arrowheads). Therefore, in peroxisomes of the *pay32* mutants, a carboxyl-terminal 45-kD fragment reactive with anti-SKL antibodies is protected by the peroxisomal membrane, while an amino-ter-

minimal 17-kD fragment is at least partially exposed to the cytoplasm, making it accessible to the action of external protease.

Our data suggest that mutations in the *PAY32* gene affect the translocation of the bulk of the peroxisome-bound matrix proteins across the peroxisomal membrane. These matrix proteins, including one of the two anti-SKL-reactive polypeptides, appear to be trapped in the peroxisomal membrane at an intermediate stage of translocation.

### **Mutations in the *PAY32* Gene Affect to Varying Degrees the Targeting of Individual Matrix Proteins to the Peroxisome**

Immunoblots probed with anti-acyl-CoA oxidase and anti-SKL antibodies showed that acyl-CoA oxidase (Fig. 8 *a*) and a 64-kD anti-SKL-reactive polypeptide (Fig. 8 *b*, arrow) were not targeted to peroxisomes (Fig. 8 *a*, 20KgP and *PER*; Fig. 8 *b*, 20KgP) but were exclusively localized to the cytoplasm (20KgS) in the *pay32-1* and *pay32-KO* mutants. In the wild-type strain, both acyl-CoA oxidase (data not shown) and the 64-kD polypeptide were found in the peroxisomal matrix (Fig. 8 *c*, S<sub>T18</sub>, lane *wt*). The 64-kD polypeptide is apparently isocitrate lyase (Eitzen, G. A., and R. A. Rachubinski, unpublished results). The specific activity of isocitrate lyase in peroxisomes isolated from the *pay32-1* ( $16.7 \pm 3.1$  mU/mg prot) and *pay32-KO* ( $18.9 \pm 3.4$  mU/mg prot) mutants did not exceed 4% of the value ( $492.6 \pm 63.8$  mU/mg prot) determined for isocitrate lyase in peroxisomes isolated from the wild-type strain. Targeting of the matrix enzyme 3-hydroxyacyl-CoA dehydrogenase was also abolished by mutations in the *PAY32* gene (Fig. 4, middle panel). In addition, as mentioned above, a few other peroxisomal matrix proteins also were not associated with peroxisomes isolated from the *pay32-1* and *pay32-KO* mutant strains (Fig. 7 *a*, arrows). In contrast to these proteins, the targeting of catalase, malate synthase (Fig. 4, middle panel), thiolase (Fig. 6, *a* and *b*), the 62-kD anti-SKL-reactive polypeptide (Fig. 8 *b*, upward pointing arrowhead), Pay32p (Fig. 9 *b*), as well as most other peroxisomal matrix proteins (Fig. 7 *a*) were only partially reduced.

### **Pay32p Is an Intraperoxisomal Protein**

Antibodies to Pay32p specifically recognized an ~71-kD protein in whole cell extracts prepared from either the wild-type or *pay32-1* strains but not the *pay32-KO* strain (Fig. 9 *a*). The difference between the predicted molecular weight of Pay32p (66,733 D) and its empirical molecular weight (71 kD) has been reported for several other TPR-containing proteins, including p62<sup>cdc23</sup> (Sikorski et al., 1993), Pas8p (McCollum et al., 1993) and Pxr1p (Dodt et al., 1995).

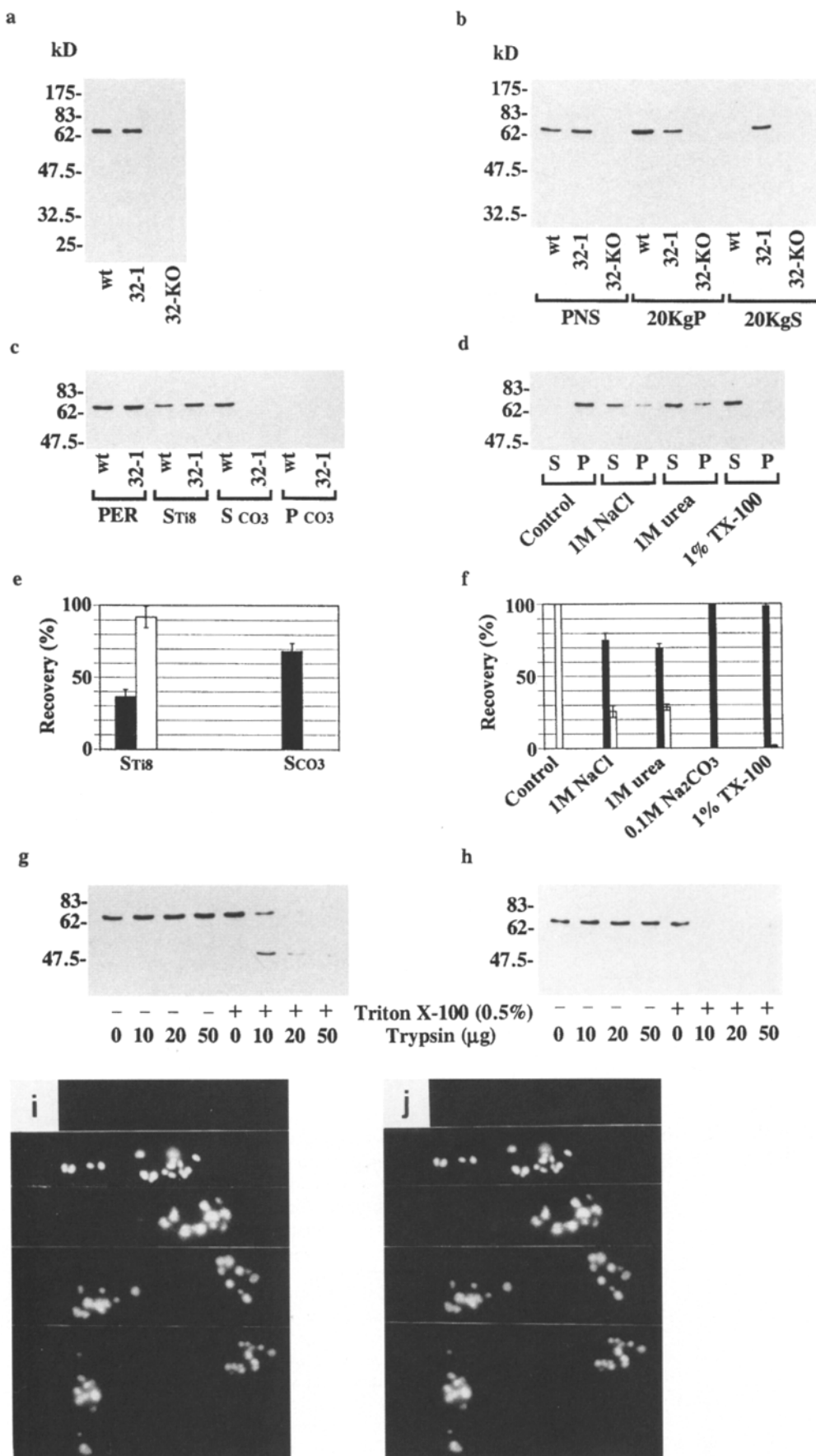
Immunoblot analysis revealed that in the wild-type strain, Pay32p was almost exclusively localized to the 20KgP enriched for peroxisomes and mitochondria (Fig. 9 *b*). Densitometric analysis showed that  $99.1 \pm 0.7\%$  of Pay32p was associated with the 20KgP. This distribution was not affected by the inclusion of NaF to the cocktail of protease inhibitors routinely added to the homogenization buffer (see Materials and Methods). Pay32p also colocalized with peroxisomes isolated from the wild-type strain by sucrose

density centrifugation (Fig. 5 *d*). The peroxisomal location of Pay32p was confirmed by double-labeling, indirect immunofluorescence analysis of wild-type cells using anti-Pay32p and anti-thiolase antibodies. The fluorescence patterns generated by these antibodies were superimposable and showed the punctate pattern characteristic of peroxisomes (Fig. 9, *i* and *j*).

Despite the fact that a large portion of Pay32p in *pay32-1* cells was localized to the cytoplasm (Fig. 9 *b*, 20KgS), the specific amount of Pay32p in highly purified peroxisomes from *pay32-1* cells was the same as in peroxisomes purified from cells of the wild-type strain (Fig. 9 *c*, *PER*, compare lane 32-1 to lane *wt*). Subfractionation of peroxisomes showed that in cells of the wild-type strain, Pay32p was associated preferentially with the peroxisomal membrane; however, ~35% of Pay32p was found in the peroxisomal matrix (Fig. 9 *c*, compare S<sub>T18</sub>, lane *wt* to *PER*, lane *wt*; Fig. 9 *e*, S<sub>T18</sub>, solid bar). In contrast, Pay32p was not associated with the peroxisomal membrane in the *pay32-1* mutant but was exclusively localized to the peroxisomal matrix (Fig. 9 *c*, compare S<sub>T18</sub>, lane 32-1 to *PER*, lane 32-1; Fig. 9 *e*, S<sub>T18</sub>, open bar). Extraction of membranes (P<sub>T18</sub>) isolated from peroxisomes of the wild-type strain with various solubilizing agents showed that Pay32p fractionated as a peripheral membrane protein that was solubilized to a large extent by either 1 M NaCl or 1 M urea (Fig. 9 *d*, lanes S; Fig. 9 *e*, solid bars) or completely by 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 11) (Fig. 9 *e*, solid bar). Protease-protection experiments revealed that in peroxisomes of both the wild-type (Fig. 9 *g*) and *pay32-1* (Fig. 9 *h*) strains, Pay32p was resistant to trypsin digestion in the absence of Triton X-100 but was degraded when the peroxisomal membrane was disrupted by the detergent. It is noteworthy that in the presence of detergent, Pay32p in peroxisomes purified from the wild-type strain was more resistant to trypsin treatment than Pay32p in peroxisomes from the *pay32-1* strain. A smaller proteolytic fragment of 52 kD was clearly detected after trypsin treatment of peroxisomes of the parental strain only, possibly representing an intermediate of proteolytic digestion (compare Fig. 9 *g* to Fig. 9 *h*). This difference in the protease sensitivity of Pay32p between the wild-type and *pay32-1* strains can be explained by a protective action of the components of peroxisomal membrane which can interact with Pay32p in peroxisomes of the parental strain but not in peroxisomes of the mutant strain.

### **Pay32p Forms a Complex with Anti-SKL-Reactive Polypeptides In Vivo**

Immunoaffinity chromatography of matrix proteins from peroxisomes of the wild-type strain showed that Pay32p and the 62- and 64-kD anti-SKL-reactive polypeptides could be coimmunoprecipitated by both anti-Pay32p and anti-SKL antibodies (Fig. 10 *a*). Therefore, Pay32p forms a complex with the anti-SKL-reactive polypeptides.  $84.6 \pm 9.7\%$  of Pay32p was present in the complex, while  $41.3 \pm 8.6\%$  of the 62-kD and  $21.5 \pm 5.4\%$  of the 64-kD anti-SKL-reactive polypeptides were associated with Pay32p (Fig. 10 *c*). The coimmunoprecipitation of Pay32p and the anti-SKL-reactive polypeptides was specific because (*a*) under native conditions, anti-SKL antibodies failed to immunoprecipitate Pay32p from the peroxisomal matrix of the



**Figure 9.** Pay32p is an intraperoxisomal protein associated with both the inner membrane surface and matrix of wild-type peroxisomes. (a) Immunoblot analysis of whole cell extracts (40 µg of protein) probed with anti-Pay32p antibodies. Strains were grown in YPBO for 9 h. (b) Immunoblot analysis of postnuclear supernatant (PNS), 20KgP, and 20 KgS fractions with anti-Pay32p antibodies. Equal portions (0.2% of total volume) of the subfractions were separated by SDS-PAGE. (c) Immunoblot analysis of whole peroxisomes (PER, 60 µg of protein) and peroxisomal subfractions with anti-Pay32p antibodies. Subfractions were prepared from 60 µg starting whole peroxisomes. (d) Immunoblot analysis of peroxisomal subfractions with anti-Pay32p antibodies. Subfractions were prepared from 60 µg starting whole peroxisomes. (e) Quantitative analysis of the distribution of Pay32p in peroxisomal subfractions of the wild-type (solid bar) and the *pay32-1* mutant (open bar) strains. Immunoblots as in c were densitometrically scanned. Values reported are standardized to the respective signals obtained for whole peroxisomes (PER) and are the means ± the standard deviation of three experiments. (f) The P<sub>Ti8</sub> from the wild-type strain was treated with various agents and divided into supernatant (S) and pellet (P) fractions as in Fig. 7 b. The control was treated with Ti8 buffer alone. S and P fractions were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Pay32p antibodies. (g) Distribution of Pay32p in the S (solid bar) and P (open bar) fractions of the wild-type and the *pay32-1* mutant strains. Immunoblots as in d were densitometrically scanned. Values reported are the means ± the standard deviation of three independent experiments. (g and h) Protease protection analysis. The P<sub>Ti8</sub> fractions from the wild-type strain (g) and the *pay32-1* mutant strain (h) were subjected to digestion with trypsin as in Fig. 6 b. (i and j) Double-labeling, indirect immunofluorescence analysis of wild-type cells using guinea pig anti-thiolase (i) and rabbit anti-Pay32p (j) primary antibodies. Primary antibodies were detected with rhodamine-conjugated donkey anti-guinea pig IgG (i) and fluorescein-conjugated goat anti-rabbit IgG (j) secondary antibodies.



*pay32-1* mutant (Fig. 10 *b*), which has no matrix-associated anti-SKL-reactive polypeptides (see Fig. 8 *c*) and (*b*) Pay32p and the anti-SKL-reactive polypeptides could be immunoprecipitated from wild-type peroxisomal matrix proteins denatured with SDS only by their respective antibodies (data not shown). As expected, Pay32p did not form a complex with either thiolase or acyl-CoA oxidase (Fig. 10 *a*).

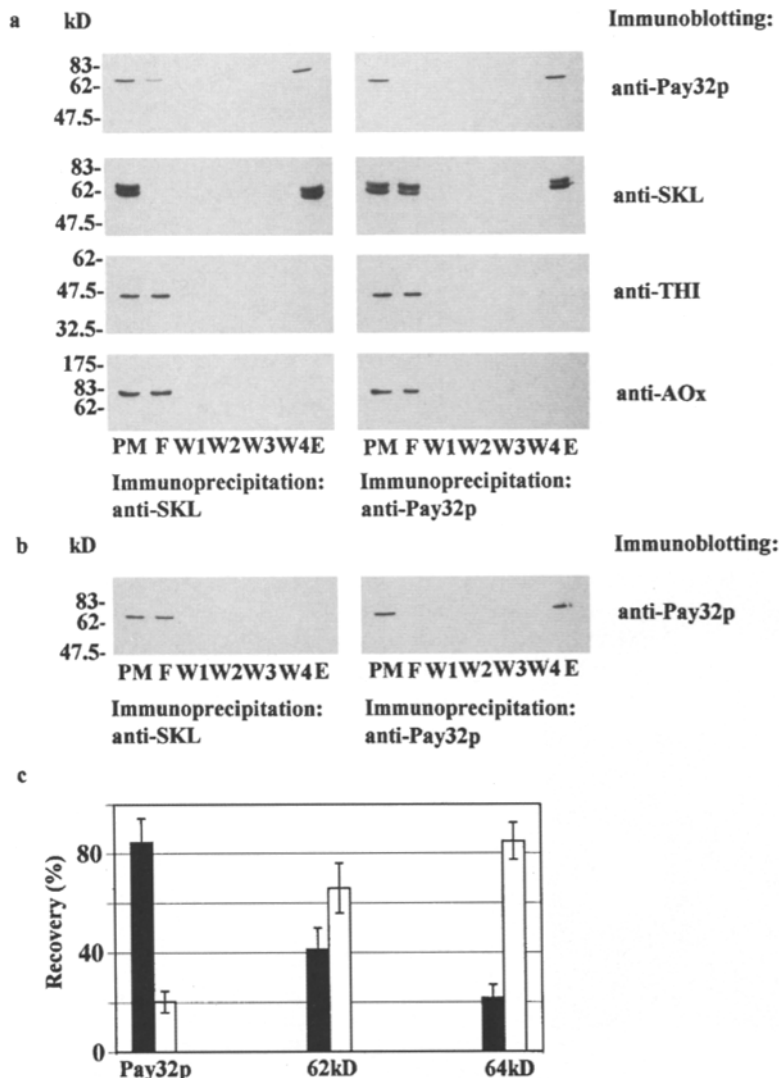
## Discussion

Here we report the isolation of *pay32* mutant strains, their biochemical and morphological characterization, the cloning and sequencing of the *PAY32* gene, and the identification and characterization of the *PAY32* gene product, Pay32p. While previous studies have revealed the existence of two different peroxisomal targeting pathways that employ different receptors to recognize proteins targeted by PTS1 (McCollum et al., 1993; van der Leij et al., 1993; Motley et al., 1994; Dodt et al., 1995; Wiemer et al., 1995; Fransen et al., 1995; Nuttley et al., 1995; van der Klei et al., 1995; Terlecky et al., 1995) or PTS2 (Marzioch et al., 1994) motifs, this study provides the first evidence that at least two distinct

translocation machineries are involved in the import of proteins into peroxisomes.

### A Subset of Proteins Is Translocated into the Peroxisomal Matrix Via a Pay32p-independent Pathway(s)

Although the targeting of thiolase, catalase, and malate synthase to the peroxisome is partially reduced by mutations in the *PAY32* gene, the peroxisome-bound forms of these proteins are translocated efficiently into the matrix in *pay32* mutants. Thiolase, catalase, and malate synthase therefore penetrate peroxisomes via a Pay32p-independent translocation machinery. *Y. lipolytica* thiolase contains an amino-terminal PTS2 (Berninger et al., 1993) like peroxisomal thiolases from other organisms (Swinkels et al., 1991; Osumi et al., 1991; Glover et al., 1994b; Erdmann, 1994). In contrast, all known peroxisomal catalases and malate synthases are targeted to peroxisomes either by variants of the carboxyl-terminal PTS1 motif or by internal peroxisomal targeting signals (de Hoop and AB, 1992; Roggenkamp, 1992; Kragler et al., 1993; Subramani, 1993). Since peroxisomal targeting signals have been evo-



**Figure 10.** Pay32p- and SKL-containing proteins form a complex in the matrix of wild-type peroxisomes. Matrix proteins ( $S_{T18}$ ) from peroxisomes of the wild-type strain (*a*) or the mutant strain *pay32-1* (*b*) were subjected to immunoaffinity chromatography with anti-SKL (*panels at left*) or anti-Pay32p (*panels at right*) antibodies linked to protein A-Sepharose. PM, peroxisomal matrix proteins (20  $\mu$ g) applied to column; F, proteins recovered in flow through; W1-W4, proteins recovered in washes; E, proteins bound to the column and eluted with 100 mM glycine (pH 2.8). Proteins were subsequently analyzed by immunoblotting with anti-Pay32p, anti-SKL, anti-thiolase (anti-THI), and anti-acyl-CoA oxidase (anti-AOx) antibodies. (*c*) Quantitative analysis of Pay32p and of the 62- and 64-kD anti-SKL-reactive polypeptides associated in a complex (*solid bar*) or present free (*open bar*) in the matrix of wild-type peroxisomes. Immunoblots as in *a* were scanned densitometrically. Values reported are standardized to the respective signals obtained for peroxisomal matrix proteins (PM) applied to the column and are the means  $\pm$  the standard deviation of three independent experiments.

lutionarily conserved (de Hoop and AB, 1992; Subramani, 1993; Purdue and Lazarow, 1994), the targeting of thiolase and of catalase and/or malate synthase probably occurs via different routes in *Y. lipolytica*. The existence of a separate targeting route for catalase in *Y. lipolytica* has been recently demonstrated (Titorenko, V. I., and R. A. Rachubinski, unpublished results). *Y. lipolytica* could use one Pay32p-independent translocation machinery to translocate proteins targeted by different PTSs via distinct receptors. However, we cannot exclude the possibility that there exists more than one Pay32p-independent translocation machinery. Further analysis of *Y. lipolytica* *pay* mutants affected in peroxisomal protein targeting and translocation should reveal which of these two models is representative.

### ***The Pay32p-dependent Translocation Machinery Acts as the Major Pathway for Protein Penetration into the Peroxisomal Matrix***

We have shown that two anti-SKL-reactive polypeptides of 62 and 64 kD are localized exclusively to the peroxisomal matrix in the wild-type strain. Only the 62-kD polypeptide was found in peroxisomes of the *pay32-1* and *pay32-KO* mutant strains, and it was exclusively associated with the peroxisomal membrane. In the mutant strains, the 62-kD polypeptide probably exists as two forms that associate with the peroxisomal membrane. One form (~30–40% of the polypeptide) behaves as a peripheral membrane protein and is apparently associated with the cytoplasmic surface of the peroxisomal membrane, perhaps through interactions with (integral) membrane proteins (see Fig. 8, *d* and *f*). The second form (~60–70% of the polypeptide) behaves as an integral membrane protein, with a 45-kD carboxyl-terminal segment apparently embedded in the peroxisomal membrane and a 17-kD segment exposed to the cytoplasm (see Fig. 8, *d*, *f*, and *h*).

Most of the proteins localized to the peroxisomal matrix in the wild-type strain are associated with the peroxisomal membrane in the *pay32* mutant strains. Some of these membrane-bound proteins behave like the peripheral form of the 62-kD anti-SKL-reactive polypeptide. They can be solubilized either at high pH or by urea, but treatment with high salt has only a minor effect on their association with the peroxisomal membrane. Other proteins can be released from the peroxisomal membrane only by treatment with detergent and therefore have solubility characteristics of the membrane-embedded form of the 62-kD polypeptide.

From our data, we can conclude that most peroxisomal matrix proteins penetrate peroxisomes via a Pay32p-dependent translocation machinery. Mutations in the *PAY32* gene prevent the translocation of peroxisome-associated forms of these proteins into the matrix. Two steps of protein penetration into the peroxisomal matrix are apparently affected in the *pay32* mutant strains: (*a*) embedding of proteins in the peroxisomal membrane and (*b*) passage of the embedded proteins through the membrane. The fraction of total protein trapped at either of these two stages can vary for different proteins. For example, 30–40% of the 62-kD polypeptide is trapped at the level of the first step, while the remainder of the polypeptide pool is primarily embedded in the peroxisomal membrane, unable to pass through it.

### ***What Role May Pay32p Have in Protein Translocation Across the Peroxisomal Membrane?***

Pay32p may function to actively pull PTS1-containing proteins into the peroxisomal matrix, because (*a*) the intra-peroxisomal pool of Pay32p is dynamically distributed between the inner surface of the peroxisomal membrane and the matrix, (*b*) the majority of Pay32p in the matrix is present in a complex with SKL-targeted proteins, which dissociate from Pay32p so as to be delivered into the matrix, and (*c*) mutations preventing the association of Pay32p with the peroxisomal membrane do not prevent the embedding of translocating proteins into the membrane but do impede their being pulled into the matrix, thereby effectively trapping the translocating proteins at the translocon. The saturation of Pay32p-dependent translocons with trapped proteins could prevent many proteins already bound to their peroxisomal receptors on the cytoplasmic surface of the peroxisomal membrane to penetrate the translocon. Indeed, a significant fraction of the proteins normally translocated via a Pay32p-dependent machinery are associated with the cytoplasmic surface of the peroxisomal membrane in *pay32* mutants. Proteins that are trapped in the peroxisomal membrane of *pay32* mutants and yet are not recognized by anti-SKL antibodies could be targeted by other variants of the PTS1 motif or could exist as intermediate complexes between PTS1-targeted and non-PTS1-targeted proteins formed normally during translocation. The existence of such complexes has been shown previously (Bellion and Goodman, 1987; McNew and Goodman, 1994).

Members of the TPR protein family essential for the import of PTS1-containing proteins have been found in different subcellular compartments: primarily cytoplasmic (*S. cerevisiae* Pas10p [Van der Leij et al., 1993]; the short form of human Pxr1p [Dodt et al., 1995; Weimer et al., 1995]), associated with the cytoplasmic surface of the peroxisomal membrane (*P. pastoris* Pas8p [McCollum et al., 1993; Terlecky et al., 1995]; the long form of human Pxr1p [Fransen et al., 1995]), and inside peroxisomes (*Y. lipolytica* Pay32p). Despite extensive sequence homology, these proteins may still perform distinct functions. Indeed, a model for the action of the short form of human Pxr1p in peroxisomal protein import proposes that this protein acts as a component of a cytoplasmic peroxisomal signal recognition particle, unlike the membrane-anchored Pas8p of *P. pastoris* or the mitochondrial presequence import receptors MAS20 and MAS70 of *S. cerevisiae*, even though these proteins are all members of the TPR family (Dodt et al., 1995; Terlecky et al., 1995). We propose that Pay32p of *Y. lipolytica* acts as an intraperoxisomal component of the matrix protein translocation machinery. An alternative point of view is that PTS1-binding TPR proteins can be organized into a highly integrated system for the import of PTS1-targeted proteins, with the members of TPR protein family being localized to different subcellular compartments. Further analysis is required to distinguish between these possibilities.

### ***How Can Defects in Pay32p Function Impair the Peroxisome Targeting and Translocation of Different Proteins in Distinct Ways?***

Mutations in the *PAY32* gene have distinct effects on the

targeting and translocation of different peroxisomal matrix proteins: (a) the targeting (binding) of thiolase, catalase, and malate synthase to the peroxisome is partially reduced, while the translocation of the peroxisome-associated forms of these proteins into the matrix appears normal, (b) the targeting of the 62-kD anti-SKL-reactive polypeptide is partially impaired, but the peroxisome-associated form of this polypeptide, as well as most peroxisomal matrix proteins, cannot be translocated across the peroxisomal membrane, and (c) the targeting of acyl-CoA oxidase, 3-hydroxyacyl-CoA dehydrogenase, and the 64-kD anti-SKL-reactive polypeptide to the peroxisome is abolished. How can we explain these data in light of our results? One scenario is that the penetration of proteins into peroxisomes via a Pay32p-dependent translocation pathway could be served by a receptor system consisting of subcomplexes with overlapping substrate specificities but varying affinities for different proteins being imported. The existence of such a receptor system has been demonstrated in mitochondria (Gratzer et al., 1995; Lithgow et al., 1995). Most peroxisomal matrix proteins, including the 62-kD anti-SKL-reactive polypeptide, would show high affinity for this receptor system. These proteins would bind to the components of the peroxisomal membrane much more quickly than would proteins with low affinity for the receptor system. As presented above, mutations in the *PAY32* gene could lead to the accumulation of proteins with high affinity on the cytoplasmic surface of the peroxisomal membrane. This accumulation could then interfere with the further binding of proteins with high affinity to the receptor complex serving a Pay32p-dependent translocation machinery, thereby causing a partial defect in their targeting. Under these conditions the targeting (binding) of proteins with low affinity to the receptor system, such as acyl-CoA oxidase, 3-hydroxyacyl-CoA dehydrogenase, and the 64-kD anti-SKL reactive polypeptide, is abolished, thereby causing their mislocalization to the cytoplasm.

While a Pay32p-independent translocation pathway may represent the main route of penetration into the peroxisomal matrix for thiolase, catalase, and malate synthase, these proteins can also use Pay32p-dependent translocons to penetrate the matrix. However, their affinity for the receptor complex serving a Pay32p-dependent translocation machinery may be low. Therefore, in *pay32* mutants the targeting (binding) of these proteins (like other proteins with low affinity) to such a receptor complex can be abolished, thereby causing a partial mislocalization of thiolase, catalase, and malate synthase to the cytoplasm.

The results described herein identify Pay32p as an intraperoxisomal component of the translocation machinery for most but not all peroxisomal matrix proteins and provide direct evidence for the existence of multiple pathways of protein translocation across the peroxisomal membrane.

R. K. Szilard is the recipient of a Studentship from the Medical Research Council (MRC) of Canada. R. A. Rachubinski is a MRC Scientist.

This work was supported by a grant from the MRC to R. A. Rachubinski.

Received for publication 5 June 1995 and in revised form 24 August 1995.

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